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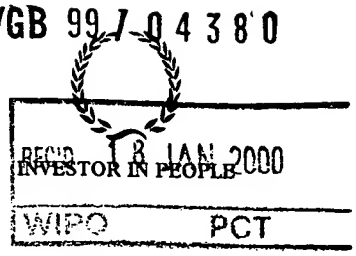

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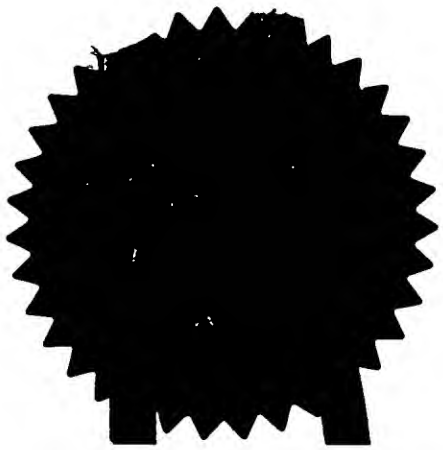

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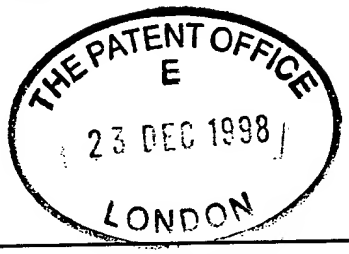

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	Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its incorporation	**BELGIUM**
4.	Title of the invention	**AN ASSAY FOR GENOTYPING CYTOCHROME EXPRESSION**
5.	Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**BOULT WADE TENNANT** **27 FURNIVAL STREET** **LONDON** **EC4A 1PQ**
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AN ASSAY FOR GENOTYPING CYTOCHROME EXPRESSION

The present invention is concerned with an assay and, in particular, with an assay for genotyping a polymorphism predictive of a phenotype associated with cytochrome expression, in this case CYP3A5.

The cytochrome P450 subfamily CYP3A represents one of the most important families of the P450 superfamily and play a major role in the metabolism of an ever expanding list of therapeutic compounds (23, 24). This family comprises the most abundantly expressed P450s in human livers, and is responsible for the metabolism of over 50% of all clinically used drugs, including the dihydropyridines, cyclosporin, erythromycin and barbiturates (1). Wide inter-individual variation in the metabolism of CYP3A substrates has been noted and is a factor in determining individual drug efficacy. Evidence also exists for the metabolism of an array of lipophilic environmental pollutants, including the activation of pro-carcinogens such as aflatoxin B1 by members of this subfamily (2).

Presently, four CYP3A cDNAs have been identified in humans, CYP3A3, CYP3A4, CYP3A5 and CYP3A7. It is believed that CYP3A3 represents an allelic variant of CYP3A4, whilst CYP3A4 and CYP3A7 are found only in human adult and fetal livers respectively (3). Initial experiments suggested that a polymorphism existed in *CYP3A4* (4). However other studies, whilst confirming a wide range of inter-individual variation in *CYP3A4* expression have failed to confirm the original bimodality (5, 6). Overlapping substrate specificities between CYP3A4 and CYP3A5 have previously made it difficult to separate metabolism by these isoforms;

consequently little phenotyping data have been produced to study variation in CYP3A5 activity in humans. However, there is evidence for the polymorphic expression of CYP3A5. Use of both immunoblotting and Northern analysis have detected CYP3A5 expression in only 10-30% of human livers (7, 8, 9). More recently, analysis of 30 human liver samples using immunoblotting found that only 3% showed no detectable CYP3A5, whilst a large number had trace amounts, suggesting that a polymorphism in this enzyme may be regulatory as opposed to structural (10). Comparisons of the 5' flanking regions from the *CYP3A4*, *3A5* and *3A7* genes have identified putative binding sites for several transcriptional regulatory factors common to all isoforms (11, 12, 13). However, the molecular basis, if any, for this inter-individual variation in expression of the CYP3A sub-family members has so far remained unclear. Indeed it has been suggested that the host cellular environment may be a greater determinant of inducibility than gene structure (14). However, the determination of a major genetic component to variant expression and activity, linked to an easy screening method, would be extremely beneficial, not only in providing a predictor of individual response to drugs which are metabolised by these isoforms, but also in facilitating association studies between *CYP3A* and disease processes.

The delineation of CYP3A4 and CYP3A5 metabolism has been shown to be possible using the sedative midazolam as a probe drug (15). In this case two metabolites are formed, 1-hydroxy midazolam (1-OHM) and 4-hydroxy midazolam (4-OHM). Those samples containing a higher proportion of CYP3A5 compared to CYP3A4 have their metabolism driven towards the 1-OHM route and

therefore show a higher ratio of 1-OHM/4-OHM than those containing only CYP3A4. The present inventors have now established that two polymorphisms, located in putative transcriptional regulatory regions, which cause increased *CYP3A5* gene expression and metabolic activity are linked and have developed PCR based assays for their detection. These assays will allow prediction of inter-individual variability in response to drugs metabolised by this isoform, as well as facilitating disease association studies.

Therefore, according to a first aspect of the present invention there is provided a method of identifying whether a sample containing DNA has a high or low drug metabolising phenotype associated with cytochrome CYP3A5 expression, which method comprises the steps of 1) providing a sample containing DNA, and 2) identifying the presence or absence of a mutation in a transcription regulatory region, such as, a promoter or enhancer adjacent the region encoding CYP3A5, using a reagent capable of distinguishing the presence or absence of a nucleotide in said regulator site. Preferably, the mutation occurs in a recognition site for a transcription factor of said regulatory region, and even more preferably in an activator protein-3 motif or a basic transcription element. Preferably, the mutation occurs at any one of positions -475 or -147 of the DNA of the 5' flanking region adjacent to the region encoding CYP3A5 the sequence of which flanking region is illustrated in Figure 7. Preferably, both the mutations at positions -475 and -147 are present.

In one embodiment of the method of the invention said sample DNA is amplified, preferably by the polymerase

chain reaction using oligonucleotide primers which are capable of hybridising selectively to the wild type sequence or primers which are capable of hybridising to the mutant sequence, such that generation of amplified DNA from said primers will indicate whether said wild type or mutation is present. In this method the PCR primers hybridise either to the mutated or wild type sequence, but not both. Amplification of the DNA of the respective mutation or wild type genotype using the respective primers will provide an indication of the presence of the wild type or mutated nucleotide mutations.

A further method of the invention advantageously utilises primers which, in addition to hybridising to the site of interest, are capable of introducing a restriction site which is absent in either the wild type or mutated sequence. Therefore, according to a further aspect of the invention, there is provided a method of identifying a mutation in a transcription regulatory region adjacent to the DNA encoding cytochrome CYP3A5, which method comprises 1) subjecting the sample DNA to the polymerase chain reaction using oligonucleotide primers which are capable of hybridising to the wild type sequence and/or to the mutation sequence at a location being analysed, which primers are such that they can introduce a restriction site at said location which is not present in the wild type or mutation sequences, and 2) subjecting amplified DNA from step 1 to a restriction enzyme which cleaves at said restriction site to provide a restriction digest indicative of the presence or absence of said mutation.

According to this aspect of the invention, the

invention occurs in a recognition site for a transcription factor of said regulatory region and preferably in an activator protein-3 motif (AP-3) and/or a basic transcription element. Preferably, the mutation occurs at any of position -475 or -147, of the regulatory region, the sequence of which is illustrated in Figure 7.

The mutations at the positions identified in each of the methods according to the invention comprise $T_{-475} \rightarrow G$ and $A_{-147} \rightarrow G$. Preferably, the primer which is used to detect the mutation at $A_{-147} \rightarrow G$ is capable of introducing a restriction site for the enzyme *Tai* I only when the wild type A nucleotide is present at position -147. Alternatively, the primer used to detect the $T_{-475} \rightarrow G$ nucleotide mutation is capable of introducing a restriction site for the enzyme *Alu* I only when the wild type T nucleotide is present at position -475.

In this embodiment an example of suitable primers is any of 3A5F1 GGGTCTGTCTGGCTGCGC
and 3A5F2(GGGGTCTGTCTGGCTGAGC)
and 3A5R1(TTTATGTGCTGGAGAAGGACG).

Using oligonucleotide mismatch primer 3A5R1 creates a *Tai* I recognition site only when the wild type A nucleotide is present at position -147. Digestion of the 369bp product with *Tai* I yields fragments of 349 and 20bp for the wild type sequence, whilst the product remains undigested if a mutant, such as the G nucleotide, is present (Figure 2). Similarly, for the detection of the $T_{-475}G$ mutation a second oligonucleotide mismatch primer 3A5F2 may be used. This primer introduces a recognition site for the

restriction enzyme *Alu* I when the wild type T is present at position -475, digesting the product to yield fragments of 318, 33 and 18bp. This site is lost when the mutant G nucleotide is present, yielding digestion products of 336 and 33bp (Figure 3).

The DNA from the sample may be extracted using techniques which are well known in the art. Any cell of the body may be utilised to obtain the DNA sample, but preferably liver cells are used.

Also provided by the present invention is a primer of from 10 to 50 nucleotides for use in amplification of a DNA sequence to detect a wild type or mutated sequence in a 5' region adjacent the sequence encoding cytochrome CYP3A5 associated with a high or low drug metabolising phenotype which primer is capable of hybridising to a region incorporating either a mutated or wild type nucleotide at position -475 or -147 of said flanking region, such that amplification of said positions will or will not proceed from said primer according to whether or not a mutation occurs at any of said positions. Preferably, the primers comprise any of the primers 3A5F1, 3A5F2 and 3A5R1 as defined herein.

A kit may be provided according to another aspect of the invention to perform the method according to the invention. Preferably, the kit will comprise a primer as described above and even more preferably the kit will further comprise a restriction enzyme capable of distinguishing between mutated or wild type positions as defined herein. Preferably, the restriction enzyme comprises any of *Tai* I or *Alu* I.

According to a further aspect of the invention there is also provided a method of identifying toxic or mutagenic effects of a test compound, such as, a drug, toxin or procarcinogen metabolised by CYP3A5 the method comprising contacting each of a cell having a high drug metabolising phenotype and a cell having a low metabolising phenotype associated with cytochrome CYP3A5 expression, with said test compound and identifying the effects of said compound on each of said high or low drug metabolising phenotype cells or other cells sensitive to said compound. An even further aspect comprises a method of diagnosing susceptibility of an individual to a disease associated with environmental toxins or procarcinogens metabolised by CYP3A5, the method comprising the steps of 1) providing a sample containing DNA, and 2) identifying the presence or absence of a mutation in a transcription regulatory region adjacent to the DNA sequence encoding CYP3A5 using a reagent capable of distinguishing the presence or absence of a nucleotide in said regulatory site. According to this aspect of the invention, the mutation occurs in a recognition site for a transcription factor of said regulatory region and preferably in an activator protein-3 motif (AP-3) and/or a basic transcription (BTE). Preferably, the mutation occurs at any of positions -475 and -147 of the regulatory region and even more preferably at both positions where the mutation may be $T\text{-}_{475}G$ or $A\text{-}_{147}G$.

Advantageously, it is also envisaged that the regulatory region of the 5' flanking region can be used to identify or purify transcription factors which bind to the 5' region including the respective nucleotides. Thus, according to a further aspect of

the invention, there is provided a method of identifying transcription factors capable of binding to a DNA fragment from a transcription regulatory region adjacent DNA encoding cytochrome CYP3A5, said method comprising contacting said DNA fragment including said transcription regulatory region with potential transcription factors and identifying any transcription factor complexed to said DNA fragments.

Using the transcription regulatory fragment it is possible to identify compounds or agents which exhibit or exert their effect on the transcription regulatory region of CYP3A5. Thus, there is provided according to this aspect of the invention a method of identifying compounds acting on a transcription regulatory region adjacent to a DNA sequence encoding CYP3A5, the method comprising transforming a cell which is deficient in CYP3A5, with a DNA construct comprising the sequence of said regulatory region, and which regulatory region is operably linked to a sequence encoding a reporter molecule, contacting said cell with a test compound and identifying any expression of said reporter molecule.

Also provided by the invention is a method of purification of transcription factors from a sample which are capable of binding to DNA from a transcription regulatory region adjacent a DNA sequence encoding cytochrome CYP3A5, the method comprising contacting a DNA fragment including said transcriptional regulatory region with a mixture of transcription factors and identifying any complexes of said transcription factors and said fragment.

An even further aspect of the invention comprises a

method of providing a measure of activity of a transcription regulatory region adjacent to DNA encoding cytochrome CYP3A5 or alternatively a method of identifying a mutation altering the activity of the transcription regulatory region the method comprising providing a DNA construct having a fragment encoding a reporter molecule operably linked to a DNA fragment comprising said regulatory region, and introducing said construct into a cell deficient in CYP3A5 expression and monitoring for the level of expression of said reporter molecule. When the method is used to identify a mutation altering the activity of the transcription regulatory control region, the method may include the further step of comparing the levels of expression of a wild type and a mutant regulatory region.

According to each of the aspects of the invention, the regulatory region includes a mutation, preferably in a recognition site for a transcription factor of said regulatory region, and preferably in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE). In a preferred embodiment the mutation occur at position -475 or -147 of the region flanking the sequence encoding CYP3A5, and which region is illustrated in Figure 7. Preferably, both the mutations are present which preferably comprise $T_{-475}G$ and $A_{-147}G$.

The methods of the present invention will be particularly valuable to establish, prior to treatment with a drug, whether the drug will be effectively metabolised by the patient.

The invention may be more clearly understood by the

following example with reference to the accompanying drawings wherein

Fig. 1a: is an illustration of the relationship between midazolam metabolic ratio and genotype for the linked $A_{-147}G$ and $T_{-475}G$ mutations in the 5' flanking region of the *CYP3A5* gene. Midazolam metabolic ratio = 1-OHM/4-OHM, wt = samples with the wild type sequence in the 5' flanking region as previously published (11), Het = samples heterozygous for the linked polymorphisms, $A_{-147}G$ and $T_{-475}G$.

Fig. 1b: is an illustration of the relationship between CYP3A5 mRNA expression and the linked $A_{-147}G$ and $T_{-475}G$ mutations in the 5' flanking region of *CYP3A5*. Relative Ct difference = difference in threshold cycle between samples, as described in the methods section wt = samples with the wild type sequence in the 5' flanking region as previously published (11) Het = samples heterozygous for the linked polymorphisms, $A_{-147}G$ and $T_{-475}G$.

Fig. 2a: is a diagram of relative position of primers, and of the recognition site for the restriction enzyme *Tai* I, which is introduced into the PCR product utilising mismatched primer 3A5R1 when the wild-type "A" nucleotide is present at position -147, and is lost when the mutant "G" nucleotide is present.

Fig. 2b: is a diagrammatical representation of expected restriction fragments for each possible genotype for the $A_{-147}G$ mutation, i.e. homozygous wild-type, heterozygous and homozygous mutant.

Fig. 2c: is an illustration of a 1.5% agarose gel of *Tai* I restriction digest of 3A5F2/3A5R1 PCR product for detection of the $A_{-147}G$ mutation. Lane 1. 100 bp ladder. Lanes 2 & 7. Reference undigested PCR products. Lane 3. Sample homozygous for the wild-type "A" nucleotide at position -147. Lanes 10, 11, 16. Samples heterozygous for the $A_{-147}G$ mutation.

Fig. 3a: is a diagram of relative position of primers, and of the recognition sites for the restriction enzyme *Alu* I. The forward recognition site is introduced into the PCR product utilising mismatched primer 3A5F2 when the wild-type "T" nucleotide is present at position -147, and is lost when the mutant "G" nucleotide is present.

Fig. 3b: is a diagrammatical representation of expected restriction fragments for each possible genotype for the $T_{-475}G$ mutation, i.e. homozygous wild-type, heterozygous and homozygous mutant.

Fig. 3c: is an illustration of a 12.5% polyacrylamide ExcelGel of *Alu* I restriction digest of the 3A5F2/3A5R1 PCR product for detection of the $T_{-475}G$ mutation. Lane 1.

100 bp ladder. Lanes 2, 5, 6, 7 & 8. Samples homozygous for the wild-type "T" nucleotide at position -147. Lanes 3, 4, 9. Samples heterozygous for the $T_{-475}G$ mutation. Fragment X - additional digestion product resulting from re-amplification of original template by primers 3A51/3A52.

Fig. 4a: is a comparison of 1-OHM/4-OHM metabolic ratios between samples with the linked mutations (HET group) and those wild-type for the mutations at positions -147 and -475 (WT group). Mean and quartiles are shown for each group, as is overall mean for the combined groups (central line).

Fig. 4b: is a comparison of CYP3A5 expression (ln transformed) between samples with the linked mutations (HET group) and those wild-type for the mutations at positions -147 and -475 (WT group). Mean and quartiles are shown for each group, as is overall mean for the combined groups (central line).

Fig. 5: is a Western blot analysis of CYP3A5 protein expression in liver samples. A Western blot of microsomes prepared from liver samples and probed with a CYP3A5 specific antibody. Liver samples containing the linked polymorphisms at -147 and -475 (wt group) are marked * (sizes indicated in kDa from Wide Range Colour Marker (signs).

Fig. 6: is a list of oligonucleotide mismatch primers used in accordance with the

invention, where the underlined nucleotide indicates the sequence mismatch.

Fig. 7: is an illustration of the nucleotide sequence of the 5' flanking region relative to the DNA sequence encoding CYP3A5.

Experimental Procedures

Liver microsome preparation

Human liver samples were obtained from kidney transplant donors, and flash-frozen immediately on removal. Human liver microsomes were prepared according to previously described protocols (21), and protein content was determined by the method of Lowry as modified by Miller (22).

Midazolam hydroxylase assay

The rates of midazolam overall metabolism and of the formation of 1- and 4-OH-midazolam were determined as follows. Each incubation vessel contained an aliquot of the microsomal suspension (containing 1 mg of microsomal protein) in 1.15 % KCl - 0.01 M phosphate buffer pH 7.4; 10 µl of a stock solution of 6 mM midazolam dissolved in DMSO to reach a final midazolam concentration of 60 µM; 500 µl of a co-factor mixture containing 0.5 mg of glucose-6-phosphate, 0.5 mg of $MgCl_2.6H_2O$, 0.5 units of glucose-6-phosphate dehydrogenase dissolved in 0.5 M Na-K-phosphate buffer, pH 7.4 and a 1.15 % KCl - 0.01 M phosphate buffer pH 7.4 to bring the incubation volume to 0.9 ml. After a pre-incubation for 5 min at 37°C, the incubations were started by adding 100 µl of a

solution of 1.25 mg/ml NADP to reach a final concentration of 0.125 mg/ml. Tubes were continuously shaken at 100 oscillations/min in an Heto shaking waterbath. Blank incubates with boiled microsomes were incubated under identical conditions as the control incubates. The incubations were stopped after 30 min by immersing the tubes in dry ice. Samples were stored at $\leq$ -18°C until analysis. The incubation samples were analysed for unchanged midazolam and for its metabolites 1'- and 4-hydroxymidazolam by HPLC with UV-detection.

HPLC determination of midazolam metabolites

The 1-ml samples of midazolam were thawed and diluted with 1 ml DMSO. Samples were sonicated for 10 min, centrifuged and an aliquot of the supernatant was injected directly onto the HPLC-column. The HPLC apparatus consisted of a Waters 600 MS pump. The samples were injected automatically, using a WISP 717 plus automatic injector. Stainless steel columns (30 cm x 4.6 mm i.d.) were packed with Kromasill 18 (5 μm) bound phase by a balanced density slurry procedure (Haskel DSTV 122-C pump, 10^7 Pa). UV-detection at 230 nm was performed using a Waters 996 Diode Array Detector. Elution at 1-ml/min started with a short gradient from 100% 0.1 M ammonium acetate, pH 7.0 (solvent system A) to 50% of solvent system A and 50% of solvent system B containing 1M ammonium acetate pH 7.0, methanol and acetonitrile (10/45/45), over a 1-min period, followed by a second gradient to 100% solvent system B in 15 min. This solvent composition was held for 2 min before equilibration with the starting conditions. The identity of the metabolites

of midazolam was confirmed using mass spectroscopy. The conversion of UV-peak areas into ng was performed by a Millennium 2020 CDS system on a calibration curve of midazolam. This calibration curve was made up after injection of known amounts of the drug (0, 1059, 2117, 3176 and 5028 ng) and linear (weighted by 1/x) regression analysis of the corresponding UV-peak areas. The equation of the calibration curve was ng = 0.000333 x area (r^2 = 0.9997, n = 5). The metabolic activity was expressed as pmol metabolite formed/min mg protein, and a metabolic ratio was determined for each sample according to the ratio of 1OHM/4OHM in each sample.

Genomic DNA preparation

DNA was isolated from frozen liver samples using a QIAmp Tissue Kit (QIAGEN) in accordance with the manufacturer's instructions.

RNA preparation

RNA was isolated from the liver samples using a QIAGEN RNAeasy Midi Kit (QIAGEN), according to manufacturers instructions. Twenty μg of RNA was treated with RNAse-free DNAse I (Boehringer Mannheim), for 30 min at 37°C in 20 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$. Samples were phenol/chloroform extracted, precipitated and resuspended in 30 μl of TE buffer. Two and a half μg of the treated sample was reverse transcribed for 50 minutes at 42°C in 1 x first strand buffer, 0.01M DTT and 0.5M dNTPs using 0.5 μg of oligo(dt) random primers and 200 units SuperScript II Reverse Transcriptase (GibcoBRL) for use on the ABI Prism 7700

Sequence Detection System (SDS).

Sequencing of the CYP3A5 5' flanking region

A 1343 bp 5' flanking region of *CYP3A5* was PCR amplified from genomic DNA isolated from liver samples, using primers 3A51 (5'-GGAAGCAACCTACATGTCCATC) and 3A52 (5'-ATCGCCACTTGCCTTCTTC) based on the published sequence of Jounaidi *et al.* (11). PCR conditions were 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 1 min, 57°C for 30 sec, 72°C for 2.5 min, and 1 cycle of 72°C for 10 min. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN), sequencing primers were designed (Table 1), and used to directly sequence the PCR product on both sense and antisense strands by cycle sequencing using the ABI BigDye Terminator cycle sequencing kit (Perkin Elmer). Sequencing reactions were analysed on an ABI 377 automated sequencer. Contig sequences were aligned and compared using the Sequence Editor version 1.0.3 software packages (Perkin Elmer) and manually edited for identification of heterozygote positions.

PCR detection assays for the $A_{-147}G$ and $T_{-475}G$ mutations

All PCR assays were performed utilising a 1 in 100 dilution of the original 3A51/3A52 PCR product as template, under the following conditions: 1 cycle of 95°C for 1 min, 30 cycles of 95°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and 1 final cycle of 72°C for 10 min. All products were sequenced to confirm the identity of the product as CYP3A5. Oligonucleotide mismatched primers utilised in the assays were: 3A5F1 (5'-GGGTCTGTCTGGCTGCGC), 3A5F2 (5'-

GGGGTCTGTCTGGCTGAGC), and 3A5R1 (5'-TTTATGTGCTGGAGAAGGACG), where positions of mismatches are underlined.

For the $A_{-147}G$ mutation, PCR was performed using primer pair 3A5F2 and 3A5R1. Twenty μl of PCR product was digested for a minimum of 3 hours at 65°C using 15 units of *Tai* I, and the restriction fragments visualised by ethidium bromide staining after electrophoresis on a 1.5% agarose gel.

For the $T_{-475}G$ mutation, PCR was performed using primer pair 3A5F2 and 3A5R1 as described above. Twenty μl of PCR product was digested with 15 units of *Alu* I for a minimum of 3 hours, and restriction fragments were separated by electrophoresis on a 12.5% ExcelGel on a Pharmacia Multiphor Electrophoresis system (Pharmacia). Fragments were visualised by silver staining in a Hoeffer Automatic Gel Stainer (Pharmacia).

To detect the presence of mutations on the same chromosome, PCR was performed using primers 3A5F1 and 3A5R1. Twenty μl of PCR product was digested for a minimum of 3 hours at 65°C using 15 units of *Mvn* I, and the resulting restriction fragments were visualised by ethidium bromide staining after electrophoresis on a 1.5% agarose gel.

Relative quantification and comparison of CYP3A5 RNA

Relative levels of CYP3A5 mRNA were determined by real time PCR using the ABI 7700 SDS (Perkin Elmer). Optimal primers and probes for the detection of CYP3A5 were designed using the PrimerExpress program, and

subsequently checked to ensure specificity for CYP3A5. Primers utilised for the quantification PCR were: forward - 5'-AAGTGGCGATGGACCTCATC-3'; reverse - 5-GAGGAGCACCAGGCTGACA-3'. The TaqMan probe was labelled with the 5' reporter dye 6-carboxy-flouresine (FAM), and had the sequence 5'-CAAATTTGGCGGTGGAAACCTGGC-3'. Optimal primer/probe ratios and concentrations were determined and the experiments run according to standard protocols for the ABI 7700 Standard Detection System. CYP3A5 mRNA expression for all samples was normalised against the expression of ß-actin mRNA. The threshold cycle (Ct) is the PCR cycle number where the ABI 7700 begins to detect an increase in fluorescent signal associated with the linear amplification of PCR product. The Ct value is dependent on the initial amount of template copy. Quantities of CYP3A5 in each sample were determined by averaging the Ct from 3 separate PCR reactions of each sample. Relative differences in Ct between samples were calculated by subtracting the Ct of each sample from the highest Ct within the samples (lowest expression). Since the amount of PCR product doubles with every cycle in the linear range of a PCR the differences in Ct were converted into estimated differences of mRNA quantity between the samples by calculating $2^{\delta Ct}$, where δCt is the difference in cycle threshold between two samples.

Negative controls were performed on each run to ensure that no signals were due to DNA contamination. Control samples consisted of RNA samples which had been treated in exactly the same manner as for the quantitative PCR, but without the addition of the reverse transcriptase.

Statistical Analysis

Statistical analysis was performed on the JMP Statistical program version 3.2.2 (SAS Institute Inc.). Metabolic ratio and CYP3A5 mRNA expression data were checked to ensure that they conformed to a normal distribution. CYP3A5 mRNA expression data did not conform to a normal distribution and were ln-transformed, afterwhich the data was normally distributed. Metabolic ratios and expression levels were compared between groups using a t-Test.

Western Blot Analysis

Forty micrograms of microsomal protein prepared from each liver were solubilised in an equal volume of Laemmli sample buffer (Biorad) by four cycles of freezing and boiling for 10 minutes. Samples were loaded onto pre-cast 10% SDS-PAGE Ready Gels (Biorad) and electrophoresed for 1 hour at 180 V. Separated proteins were transferred onto Hybond-P membranes (Amersham) using a Trans-blot SD apparatus (Biorad). Membranes were blocked by an overnight incubation at 4°C in 1x PBS containing 5% (w:v) nonfat milk and 0.1% (v:v) Tween. Membranes were incubated at ambient temperature for 1 hour in a 1:3000 dilution of specific human CYP3A5 antibody (Gentest) in 1X PBS, 2.5% nonfat milk, then rinsed four times in 1x PBS, 2.5% (w:v) nonfat milk, 0.1% (v:v) Tween. Membranes were incubated at ambient temperature for 1 hour in a 1:5000 dilution of Anti-Rabbit IgG peroxidase conjugate (Sigma) in 1x PBS, 2.5% (w:v) nonfat milk, and rinsed as previously. The membranes were developed using the ECL Plus Western Blotting Detection System (Amersham) according to

manufacturer's instructions, and visualised by autoradiography using Kodak X-Omat film (Sigma).

Example 1

Midazolam phenotyping

A panel of 39 liver samples was phenotyped for CYP3A5 activity, using the metabolism of midazolam to its 1-OH metabolite as a marker of activity. Human liver microsomal samples containing CYP3A5 in addition to CYP3A4 exhibit a significantly greater ratio of 1-OHM to 4-OHM compared with samples containing only CYP3A4. 1-OHM/4-OHM ratios between 5 and 9 were observed for microsomes containing both CYP3A4 and CYP3A5. Samples containing only CYP3A4 showed 1-OHM/4-OHM ratios < 4 (15). Analysis of the CYP3A5 phenotypes in our data set showed a clear bimodal distribution, with 6 samples (15%) having metabolic ratios greater than 5, and the remaining samples having metabolic ratios lying between 1.5 and 3.5 (see Fig. 1a). Of the 39 liver samples from which microsomes were prepared for metabolic analysis, sufficient tissue was available for full DNA and RNA analysis for 26, which included 6 samples lying in the higher metabolic ratio range. In addition to these 26 samples microsomes for protein analysis were available for a further 3 samples, all of which had metabolic ratios of <4.

Analysis of CYP3A5 gene 5' flanking region

The 5' flanking region of CYP3A5 was PCR-amplified from genomic DNA of all 26 samples and sequenced in full, as shown in Figure 7. Alignment showed that the region was well conserved. Only a small number of

inter-individual variations were identified in addition to a few variations from the published sequence (Table 2.). All variants detected were heterozygous, and all samples heterozygous for the more frequent $A_{-147}G$ mutation were also heterozygous for the $T_{-475}G$ mutation, suggesting that the two mutations were linked. These two mutations fall within two separate putative regulatory elements, a basic transcription element (BTE: $A_{-147}G$) and an activator protein-3 motif (AP-3: $T_{-475}G$). None of the remaining variants fell within putative regulatory domains.

PCR assays were developed to confirm the presence of the $A_{-147}G$ and $T_{-475}G$ mutations individually, and to ascertain if the two mutations were on the same, or on separate chromosomes. The PCR assay for the $A_{-147}G$ mutation was based on the creation of a recognition site for the restriction enzyme *Tai* I by utilising an oligonucleotide mismatch primer (3A5R1). This primer introduces a *Tai* I recognition site only when the wild-type "A" nucleotide is present at position -147. Digestion of the 369bp product with *Tai* I yields fragments of 349 and 20bp for the wild-type sequence, whilst the product remains undigested if the mutant "G" nucleotide is present (Fig. 2). Similarly, for the detection of the $T_{-475}G$ mutation a second oligonucleotide mismatch primer was used (3A5F2). This primer introduces a recognition site for the restriction enzyme *Alu* I when the wild-type T nucleotide is present at position -475, digesting the product to yield fragments of 318, 33 and 18 bp. This site is lost when the mutant G nucleotide is present, yielding digestion products of 336 and 33 bp (Fig. 3).

To determine if the mutations were present on the same chromosome a PCR assay was developed utilising two oligonucleotide mismatch primers (3A5F1 and 3A5R1), both primers introducing recognition sites for the restriction enzyme *Mvn* I when the mutant nucleotides are present at positions -147 and -475. If the mutations are present on the different chromosomes then the original 369 bp product is digested to yield products of 349/350 bp and 20/19 bp (inseparable by gel electrophoresis), whilst if present on the same chromosome the fragment is digested to yield products of 330 and 20/19 bp (data not shown). In addition to confirming the individual genotypes of the samples as determined by sequencing the two mutations were, in all cases, linked on the chromosome (data not shown).

Relationship between CYP3A5 allelic variants, CYP3A5 mediated metabolism, CYP3A5 mRNA and protein expression

Samples were grouped according to genotype: "wild-type" or "mutant" (containing the linked polymorphisms), and the 1-OHM/4-OHM metabolic ratios (mr) were compared between the groups (Fig. 4a). With the exception of one outlier (liver sample number, mr = 2.08), all individuals carrying the linked mutations had metabolic ratios > 5.0, whilst the wild type group all possessed metabolic ratios of < 3.5. The mean metabolic ratios for the mutant group were significantly higher than those from the wild-type group (6.0 $\pm$ 2.0 versus 2.7 $\pm$ 0.42, mean $\pm$ standard deviation; $p < 0.001$)

Quantitative PCR was used to ascertain if the mutations in the 5' flanking region were affecting

gene expression. Whilst mRNA levels showed greater variation than the metabolic data, a degree of bimodality was observed (Fig. 1b). The mutant group had CYP3A5 mRNA levels skewed towards the higher end of the expression range, showing significantly higher levels of CYP3A5 mRNA than the wild type group (mean lnCt = 4.03, standard deviation = 0.97 , against mean lnCt = 2.06, standard deviation =1.2, $p < 0.006$) ((Fig. 4b). In this case the outlier (presenting with the mutant genotype, but wild type metabolic ratio) also fell within the lower range of expression (ln Ct = 2.9).

The level of CYP3A5 protein expression levels was determined for 29 liver samples by Western blot analysis using a CYP3A5 specific antibody. A single band of 52 kDa corresponding to CYP3A5 was clearly apparent in some samples. With the exception of the single outlier with the high expression genotype (mutant) and low metabolic ratio phenotype (wild-type), all samples which possessed the high expression genotype, a high metabolic ratio and high RNA expression level clearly show high levels of CYP3A5 expression when compared to those samples with the low expression genotype and phenotype (Fig. 5). The single outlier with the high expression genotype, but low expression phenotype showed levels of CYP3A5 expression similar to those in the low expression genotype group. Longer exposure of the Western blot indicated that a very low level of CYP3A5 expression was apparent in most samples (data not shown).

The 5' flanking sequences of CYP3A5 obtained in this study are virtually identical to those published by Jounaidi *et al.* (11), and show little inter-individual

variation in sequence. Interestingly, Jounaidi *et al.* sequenced two human genomic clones, one of which contained the two linked mutations described in detail in this report. This would suggest that one clone was derived from an individual in the low expression group, and one from an individual in the high expression/metabolism group.

Previous studies had suggested that CYP3A5 was expressed in 10-30% of livers (7, 8, 9) whilst another study has stated that some expression is constitutive in all samples (10). The present study supports the findings that some CYP3A5 expression is constitutive, with some metabolic activity and mRNA being detected in all livers studied, although CYP3A5 protein was not convincingly demonstrated in all samples using the procedures required. We detected enhanced RNA and protein expression in 23% of the samples for which tissue was available (6 out of 26), which is similar to the fraction of liver showing expression in previous studies. This supports the finding of Boobis *et al.* (10) that some show low level expression is constitutive in all liver samples although this can only be detected using more sensitive detection techniques (such as PCR, and not by Western or Northern blot analysis.

Whilst both polymorphisms detected lie within putative transcriptional regulatory elements, we suspect that the variant within the BTE is more likely to be responsible for altered expression since it has been reported that a BTE flanking the TATA box accounts for the constitutive expression of CYP1A1, and a similar region has been found in several other CYP genes including *CYP2B1*, *CYP2B2*, *CYP2E1* (16) *CYP3A4* (13) and

CYP3A7 (12). In the case of CYP3A4 gene this element has been shown to bind nuclear extracts (13) and a basic transcription element binding factor for CYP3A7 (12), pointing to a role of this region in the general control of cytochrome P450 expression. The exact mechanism of up-regulation of CYP3A5 expression in the allelic variant described here remains to be determined although the presence of one of the mutations within the BTE, and the relevance of this element for the expression of other P450s indicates a possible mechanistic link. Using methylation interference footprinting, it has been shown that all guanine residues within the BTE, and other guanine residues in the vicinity, interacted with the transcriptional factors BTEB (BTE-binding protein) and Sp1 (19). Given that the mutation within the BTE described in this paper alters an adenine residue to a guanine residue, then this could facilitate binding of transcription factors to the variant form of the BTE.

Although there is considerable overlap in the range of CYP3A5 mRNA levels seen in the homozygous and heterozygous group, the distribution of metabolic ratios is clearly bimodal, as is the amount of CYP3A5. We cannot exclude the presence of other polymorphisms that may affect the translation efficiency or protein stability of CYP3A5. But given the better correlation between DNA polymorphism and protein level and the notorious lability of RNA, the simpler explanation is that differential RNA degradation or yield (due to differences in sample handling) has blurred the distinction between high and low expressers. Whatever the explanation for the discrepancy at the mRNA level, it does not in any way diminish the predictive value of the DNA polymorphism described.

There is, however, one individual whose genotype (heterozygous mutant) is not predictive of his metabolic phenotype (low expression). The fact that CYP3A5 protein as well as mRNA levels were low in this outlier indicates that the explanation must be sought at the transcriptional level, e.g. in transcription factors controlling CYP3A5 expression.

An AUG element in the 5'- untranslated region of the BTEB gene has been shown to be, at least in part, responsible for cell specific translational control of BTEB (20). Mutations within this region were shown to affect BTEB translation. Therefore, whilst the outlier in our study has a high expression genotype for CYP3A5 expression, this individual may have a "poor" expression phenotype for BTEB. Additionally, it is possible that a mechanism similar to that responsible for inducing CYP1A1 expression may also affect CYP3A5 expression. In addition to the BTE, CYP1A1 expression is mediated by a xenobiotic responsive element (XRE). In this case inducers enhance expression by binding to a cytosolic receptor (Ah receptor) which is translocated into the nucleus (possibly in association with an accessory protein coded for at the Arnt gene), where it binds the XRE (17, 18). Although variations in these and other transcription factors could further modulate CYP3A5 expression, this does not detract from the fact that the polymorphism described here seems to be the major determinant of CYP3A5 expression, at least in liver.

Despite the relatively small number of samples available for analysis in the present study, strong associations have been found between the two linked polymorphisms on the one hand and both expression and

CYP3A5 mRNA, protein and activity levels in the liver on the other hand. The unravelling of a genetic mechanisms for the polymorphic metabolism by CYP3A5 will have important consequences in the field of pharmacogenetics. The ability to predict metabolism by genotyping will greatly facilitate disease association studies and may also help to explain adverse reactions or poor response to therapeutics which are metabolised by this cytochrome P450 isoform. It will also help in delineating which factors affecting CYP3A5 activity are genetic and which are environmental; for both further work will be required to fully understand the complex variation in expression observed with this enzyme.

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Table 1. Primers used for sequencing 5' flanking region of CYP3A5 from PCR product 3A51/3A52 (see text).

Primer	Orientation #	Position*	Sequence (5'-3')
3A51	F	-1237- -1217	GGAAGCAACCTACATGTCCATC
3A5p01	F	-978- -963	AGTACAGGGAGCACAG
3A5p08	R	-917- -932	CACCTATTCATTCCTG
3A5p02	F	-698- -684	TGCTATCACCACAGAC
3A5p07	R	-689- -704	GGTGATAGCAATAGAC
3A5p03	F	-364- -349	AGGATGTGTAGGAGTC
3A5p06	R	-417- -434	CCTCACACAGATGTAACC
3A5p04	F	-176- -161	TAAGAACTCAGGTTCC
3A5p05	R	-178- -194	CAGAAACTGAAGTGGAG
3A52	R	+105- +87	ATCGCCACTTGCCTTCTTC

F = 5' to 3', R = 3' to 5'

* Primer locations are based on CYP3A5 sequence data of Jounaidi *et al* (11)

Table 2.

Position	Variant Sequence	Percentage
-475	T-K (T or G) heterozygote	30.6% (11/36)
-147	A-R (A or G) heterozygote	30.6% (11/36)

CLAIMS

1. A method of identifying whether a sample containing DNA has a high or low drug metabolising phenotype associated with cytochrome CYP3A5 expression, which method comprises the steps of:

1) providing a sample containing DNA, and

2) identifying the presence or absence of a mutation in a transcription regulatory region adjacent to the sequence encoding CYP3A5 using a reagent capable of distinguishing the presence or absence of a nucleotide in said regulatory region.

2. A method according to claim 1 wherein said mutation occurs in a recognition site for a transcription factor of said regulatory region.

3. A method according to claim 1 or 2 wherein said mutation occurs in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE).

4. A method according to any of claims 1 to 3, wherein said mutation occurs at any one of positions -475 or -147 of the transcription regulatory region adjacent to the sequence encoding CYP3A5 the sequence of which region is illustrated in Figure 7.

5. A method according to any of claims 1 to 4 wherein both mutations at position -475 or -147 are present.

6. A method according to any of claims 1 to 5 wherein said sample DNA is amplified, preferably by the polymerase chain reaction, using oligonucleotide

primers which are capable of hybridising selectively to the wild type or mutant sequences respectively such that generation of amplified DNA from said respective primers will indicate whether said wild type or said mutation is present.

7. A method of identifying a mutation in a transcription regulatory region adjacent to the DNA encoding cytochrome CYP3A5 said method comprising the steps of:

1) subjecting the sample DNA to amplification, preferably by the polymerase chain reaction, using oligonucleotide primers which are capable of hybridising to the wild type sequence and/or to the mutant sequence at a location being analysed, which primers are such that they can introduce a restriction site at said location which is not present in the wild type or mutated sequences, and

2) subjecting amplified DNA from step 1 to restriction with an enzyme which cleaves at said restriction site to provide a restriction digest indicative of the presence or absence of said mutation.

8. A method according to claim 7 wherein said mutation occurs in a recognition site for a transcription factor of said regulatory region.

9. A method according to claim 7 or 8 wherein said mutation occurs in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE).

10. A method according to any of claims 7 to 9 wherein said mutation occurs at any one of positions

-475 or -147 of the transcription regulatory region adjacent to the sequence encoding CYP3A5 the sequence of which is illustrated in Figure 7.

11. A method according to any of claims 7 to 10 wherein both of said mutations at position -475 and -147 are present.

12. A method according to any of claims 1 to 11 wherein the transcription regulatory region comprises the mutations $T_{-475}\rightarrow G$ and $A_{-147}\rightarrow G$.

13. A method according to claim 12 wherein said primer for detecting $A_{-147}\rightarrow G$ is capable of introducing a restriction site for the enzyme *Tai* I, only when the wild type A nucleotide is present at position -147.

14. A method according to claim 13 said primer comprises the sequence designated 3A5R1 illustrated in Figure 6.

15. A method according to claim 12 wherein said primer for detecting $T_{-475}\rightarrow G$ is capable of introducing a restriction site for the restriction enzyme *Alu* I when the wild type T nucleotide is present at position -475.

16. A method according to claim 15 wherein said primer comprises the sequence designated 3A5F2 illustrated in Figure 6.

17. A primer of 10 to 50 nucleotides for use in amplification of a DNA sequence to detect a wild type or mutated sequence in a transcription regulatory region adjacent a sequence encoding cytochrome CYP3A5

associated with a high or low drug metabolising phenotype, which primer is capable of hybridising to a region incorporating either a mutated or wild type nucleotide in said region, such that amplification of said region will or will not proceed from said primer according to whether or not a mutation occurs in said region.

18. A primer according to claim 17 which is capable of hybridising to a recognition site for a transcription factor of said regulatory region.

19. A primer according to claim 17 or 18 which is capable of hybridising to an activator protein-3 motif (AP-3) or a basic transcription element.

20. A primer according to any of claims 17 to 19 wherein said primer is capable of hybridising to a region comprising a mutation at any of position -475 or -147 of the transcription regulatory region, illustrated in Figure 7.

21. A primer according to any of claims 17 to 20 which comprises any of the sequences designated 3A5F1, 3A5F2 or 3A5R1 illustrated in Figure 6.

22. A kit for performing the method of any of claims 1 or 7 comprising a primer according to any of claims 17 or 21.

23. A kit according to claim 22 further comprising a restriction enzyme capable of producing a restriction digest for distinguishing between said mutated or wild type sequences.

24. A kit according to claim 23 wherein said enzyme comprises any of *Tai* I or *Alu* I.

25. A method of identifying toxic or mutagenic effects of a test compound, such as, a drug, toxin or procarcinogen metabolised by CYP3A5 the method comprising contacting each of a cell having a high drug metabolising phenotype and a cell having a low metabolising phenotype associated with cytochrome CYP3A5 expression, with said test compound and identifying the effects of said compound on each of said high or low drug metabolising phenotype cells or other cells sensitive to said compound.

26. A method of diagnosing susceptibility of an individual to a disease associated with environmental toxins or procarcinogens metabolised by CYP3A5, which method comprises the steps of:

1) providing a sample containing DNA, and
2) identifying the presence or absence of a mutation in a transcription regulatory region adjacent to the sequence encoding CYP3A5 using a reagent capable of distinguishing the presence or absence of a nucleotide in said regulatory region.

27. A method according to claim 26 wherein said mutation occurs in a recognition site for a transcription factor of said regulatory region.

28. A method according to claim 26 or 27 wherein said mutation occurs in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE).

29. A method according to any of claims 26 to

28, wherein said mutation occurs at any one of positions -475 or -147 of the transcription regulatory region adjacent to the sequence encoding CYP3A5 the sequence of which region is illustrated in Figure 7.

30. A method according to any of claims 26 to 29 wherein both mutations at position -475 or -147 are present.

31. A method according to any of claims 26 to 30 wherein the transcription regulatory region comprises the mutations $T_{-475}G$ and $A_{-147}G$.

32. A method of providing a measure of activity of a transcription regulatory region adjacent to DNA encoding cytochrome CYP3A5 or of identifying a mutation changing the activity of said transcription regulatory region, the method comprising providing a DNA construct having a fragment encoding a reporter molecule operably linked to a DNA fragment comprising said transcription regulatory region, and introducing said construct into a cell deficient in CYP3A5 expression and monitoring for the level of expression of said reporter molecule.

33. A method of identifying transcription factors capable of hybridising to a DNA fragment from a transcription regulatory region adjacent to DNA encoding cytochrome CYP3A5, said method comprising contacting said DNA fragment including said transcription regulatory region with potential transcription factors and identifying any transcription factor complexed to said DNA fragments.

34. A method of identifying compounds acting on

a transcription regulatory region adjacent to a DNA sequence encoding CYP3A5, the method comprising transforming a cell which is deficient in CYP3A5, with a DNA construct comprising the sequence of said regulatory region, and which regulatory region is operably linked to a sequence encoding a reporter molecule, contacting said cell with a test compound and identifying any expression of said reporter molecule.

35. A method of purifying transcription factors from a sample which are capable of binding to DNA from a transcription regulatory region adjacent to a sequence encoding cytochrome CYP3A5, the method comprising contacting a DNA fragment including said transcriptional regulatory region with a mixture of transcription factors and identifying any complexes of said transcription factors and said fragment.

36. A method according to any of claims 32 to 35 wherein said transcription regulatory region includes a mutation in a recognition site for a transcription factor of said regulatory region.

37. A method according to any of claims 32 to 36 wherein said mutation occurs in an activator protein-3 motif (AP-3) and/or a basic transcription element (BTE).

38. A method according to any of claims 32 to 37 wherein said mutation occurs at any one of positions -475 or -147 of the transcription regulatory region adjacent to the sequence encoding CYP3A5, the sequence of which region is illustrated in Figure 7.

39. A method according to any of claims 32 to 38 wherein both mutations at positions -475 and -147 are present.

40. A method according to any of claims 32 to 39 wherein the transcription regulatory region comprises the mutations $T_{-475}G$ and $A_{-147}G$.

Fig 1a. CYP3A5 Genotype/Phenotype Relationship

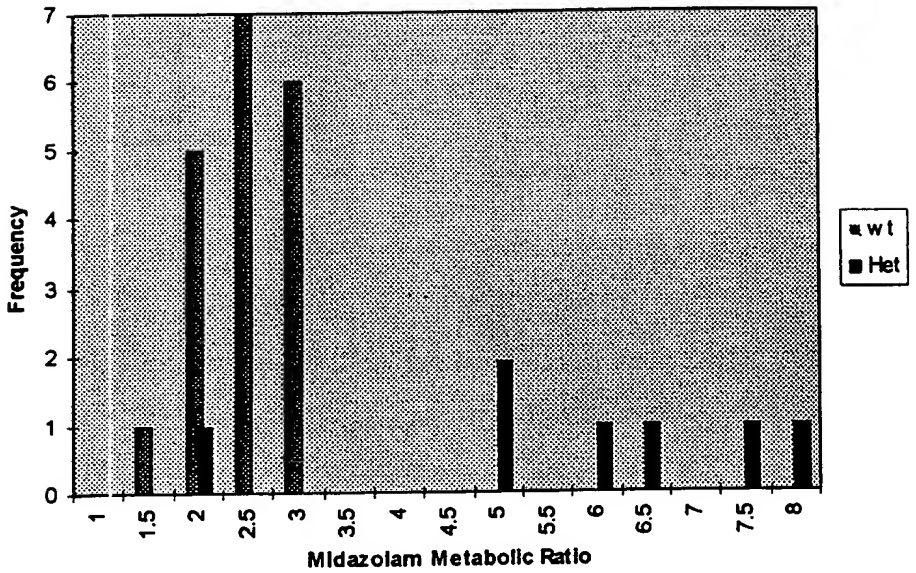
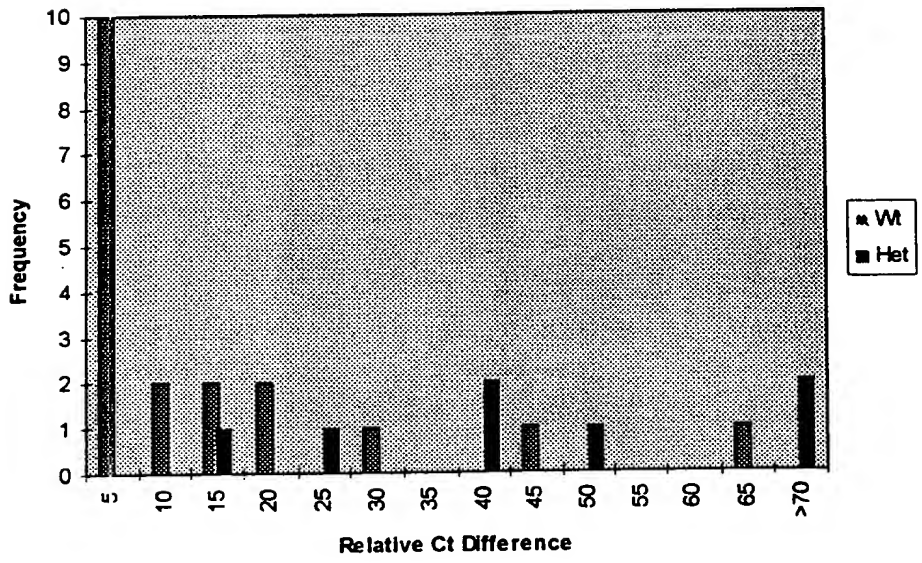
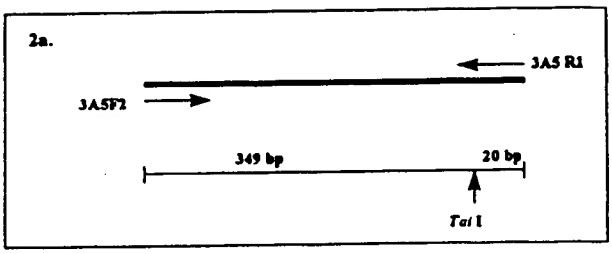
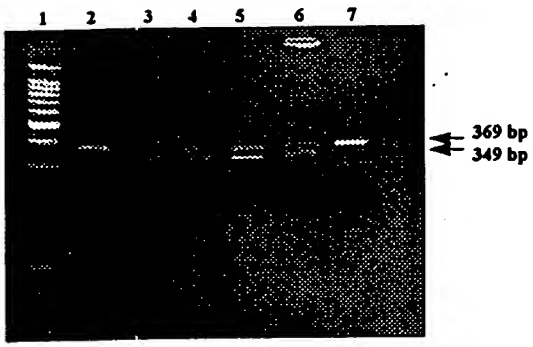


Fig. 1b. CYP3A5 mRNA Expression Related to Genotype

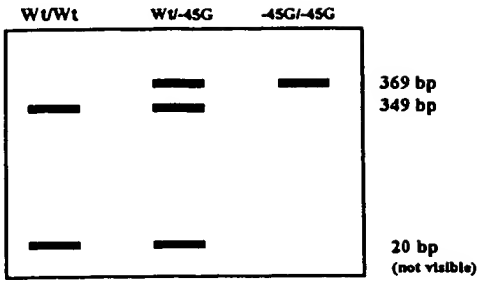


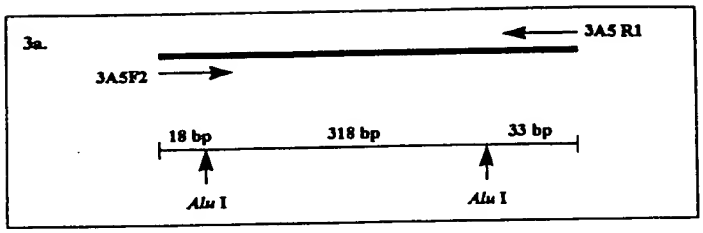

2a.
3A5 R1
3A5F2
349 bp
20 bp
TaiI

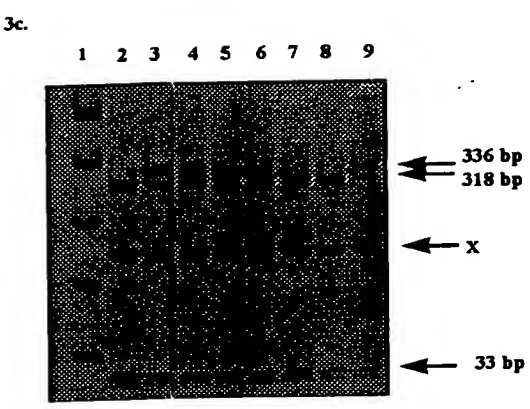
2c.


1
2
3
4
5
6
7
369 bp
349 bp

2b.


Wt/Wt
Wt/-45G
-45G/-45G
369 bp
349 bp
20 bp
(not visible)


3a.
3A5 R1
3A5F2
18 bp
318 bp
33 bp
Alu I
Alu I


3c.
1 2 3 4 5 6 7 8 9
336 bp
318 bp
X
33 bp

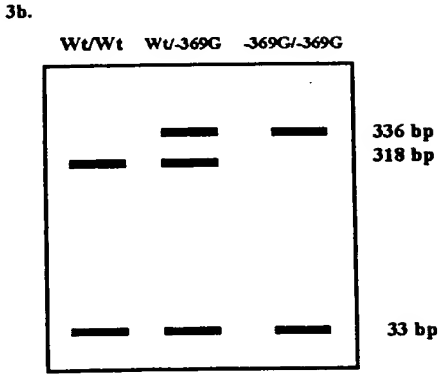

3b.
Wt/Wt
Wt/-369G
-369G/-369G
336 bp
318 bp
33 bp

Fig. 4a. Metabolic ratio by Group

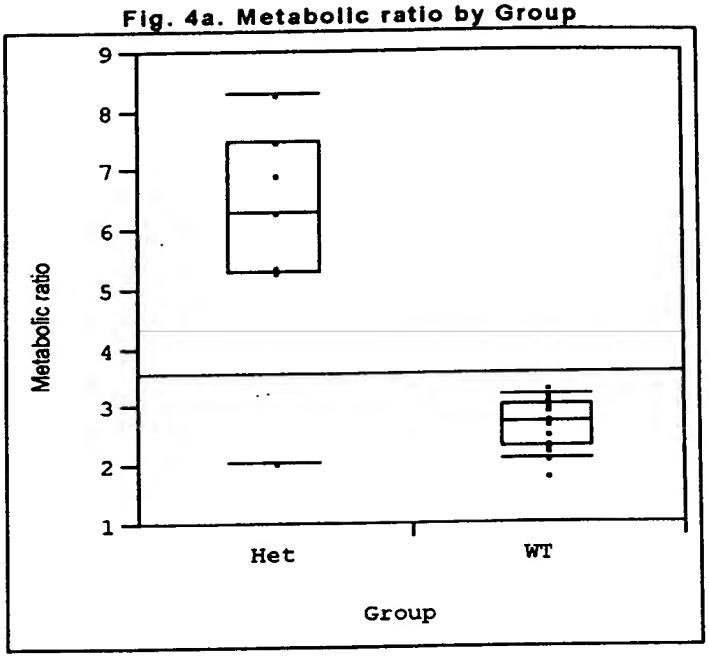
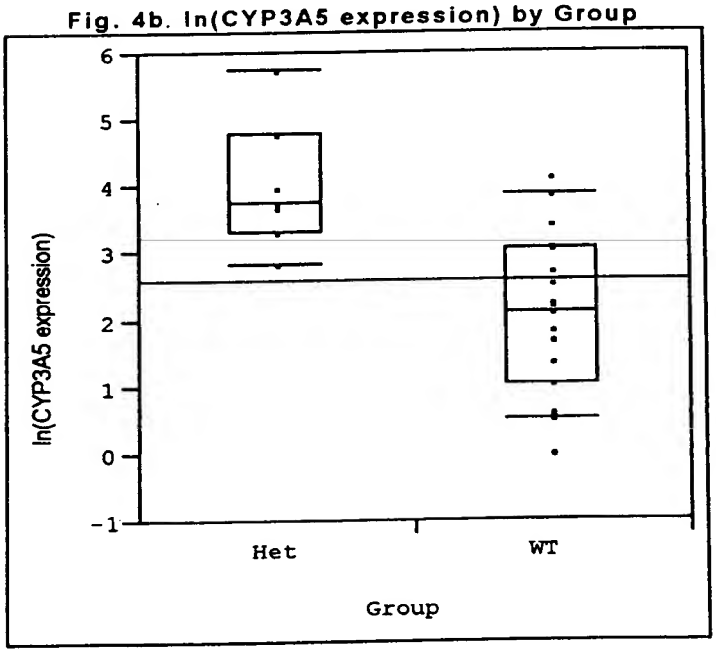


Fig. 4b. ln(CYP3A5 expression) by Group



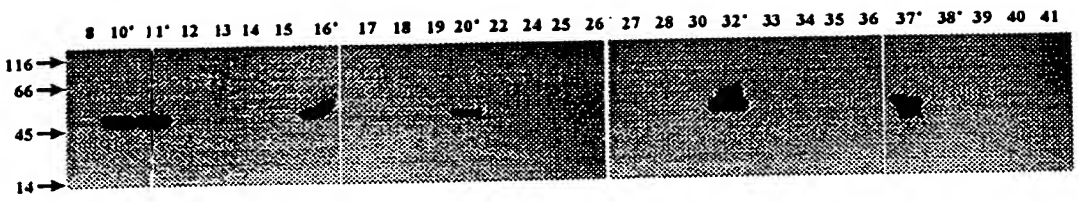

8 10* 11* 12 13 14 15 16* 17 18 19 20* 22 24 25 26 27 28 30 32* 33 34 35 36 37* 38* 39 40 41
116
66
45
14

Fig. 5

3A5F1 5'-GGGTCTGTCTGGCTGCGC-3'

3A5F2 5'-GGGGTCTGTCTGGCTGAGC-3'

3A5R1 5'TTTATGTGCTGGAGAAGGACG-3'

Fig. 6.

-1343 GGAAGCAACC TACATGTCCA TCAACAGATG AATGGGTAAA GAGAGTACTT CACTTATGCA CAATGGAGTA

-1273 CAATTCAGCC ATGAAAAAAG CATGAGATCC TGTCCTTTAT AATAACGTGG CTGGAACTGC AGGTCATTAT

-1203 GTTAGGTAAA ATAAGCCAGG CACACAAAGA CAGACATTGC ATGTTCTCAC TTATTTGTGG GATCTACAAA

-1133 TCAAAACAAT TGAGCTAATG TCTGGGTCTT AGTCAATTTT GTACCCTAAG TACAGGGAGC ACAGCCATTA

-1063 GAATACATGA TGAATGCTTT AATACAGGAA TGAATAGGTG AGAGGCACAG GGTGGTTGGG TGTTCTTCTG

-993 ATACATAGTA TCTTCCTTGA CACATTCAGT ACAACTCTCA ACAGGTAAGT CTCTTCATGT ATGTTACCTT

-923 CTGAGGAATT AAGTGGCAGA ACATGCCTTC TATTATTTTC CTTTGCAGAA CAAGACCAAT TGCATTAGTT

-853 GGGAAACAGT GCTGGCTGCA TCTGAGCCCC AAGCAACCAT TAGTCTATTG CTATCACCAC AGACTCAGAG

-783 GGGATGACAC ACAGGGGCCC AGCAATCTCA CCCAAGTCAA CTCCACCAAC ATTTCTGGTC ACCCACCATG

-713 TGTACAGTAC CCTGCTAGGG TCCAGGGTCA TGAAAGTAAA TAATACCAGA CTGTGCCCTT GAGGAACTCA

-643 CCTCTGCTAA GGGAAACAGG CACAGAAACC CACAAGGGTG GTAGAGAGGA AATAGGACAA TAGGACTGTG

-573 TGAGGGGGAT AGGAGGCACC CAGAGGAGGA AATGGTTACA TCTGTGTGAG GAGGTTGGTA AGGAAAGACT

-503 TTAATAGAAG GGGTCTGTCT GGCTGGGCTT GCAAGGATGT GTAGGAGTCA TCTAGGGGGC ACAAGTACAC

-433 TCCAGGCAGA GGGAATTGCA TGGGTAAAGA TCTGCAGTTG TGGCTTGTGG GGATGGATTT CAAGTATTCT

-363 GGAATGAAGA CAGCCATGGA AACAAGGGCA GGTGAGAGGA TATTTAAGAG GCTTCATGCC AATGGCTCCA

-293 CTTCAGTTTC TGATAAGAAC TCAGGTTCCG TGGACTCCCT GATAAAACTG ATTAAGTTGT TTATGATTCC

-223 CCATAGAATA TGAACTCAAA GGAGGTAAGC AAAGGGGTGT GTGCGATTCT TTGCTACTGG CTGCAGCTGC

Fig 7

24 DEC 1999

-153 AGCCCCACCT CCTTCTCCAG CACATAAACA TTTCAGCAGC TTGACCTAAG ACTGCTGTGC AGGGCAGGGA

-83 TGCTCCAGGC AGACAGCCCA GCAAACAACA GCACACAGCT GAAAGTAAGA CTCAGAGGAG ACAGTTGAAG

+1

-13 AAGGCAAGTG GCGATG

Fig 7 (cont)